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COACTIONS BETWEEN LITTER-DECOMPOSING HYMENOMYCETES AND THEIR ASSOCIATED MICROORGANISMS DURING DECOMPOSITION OF BEECH LITTER

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The study of a microbial community on litter should comprise not only the determination of the microflora and the estimation of the population, but also the confirmation of the various transformations of the substances in the litter and the interactions among the important microbial groups in the natural habitats.

In the previous papers on the microbial decomposition of the beech litter, studies were made first on the microbial population in the litter, and the chemical change involved in its decomposition (Saitô 1956, 1957), and next on the specific features and behaviours of the fungi playing prominent rôle in the decomposition (Saitô 1958). The particular rôle of the various groups of fungi at different stages of decomposition and the successive changes of the fungus population were outlined from the results of experimental study with the litter (Saitô 1960).

From the results hitherto obtained, for a better understanding of the mechanism of microbial succession it become desirable to know the interactions among the important microorganisms. To throw light on the detailed interrelations between the changing members of the fungi and the changes in the chemical constituents due to the fungal activities, attempts were made first to ascertain the effect of the available nutrients in single and mixed cultures with the beech leaves. Another purpose of this work is to elucidate the ecological significance of antibiosis by litter-decomposing hymenomycetes taking the main part in the microbiological decomposition of the beech litter. Because of the complicated conditions prevailing in the natural habitats, it is difficult to determine in the field what the particular important factor is and to know why. The experiments with the simplified populations in the laboratory may provide a means for the isolation of single factors and quantitative studies of the various interactions. In the natural environments, however, more factors than those detected by the experiments in the laboratory might well be affecting the microbial phenomena. The reconstructions were attempted with this limitation in mind.

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MATERIALS AND METHODS

Materials Most of the fungi and bacteria used in this study are the same as those employed in the previous works (Saitô 1958, 1960). The two predominant species of litter-decomposing hymenomycetes, *Collybia* sp. E (Pl., fig. 1, the strain designated as *Collybia* sp. in the previous papers) and *Collybia* sp. F were chosen for the study. Of the microfungi, *Absidia glauca* Hagem and *Trichoderma viride* Pers. ex Fr. were used chiefly. A species of cup fungi, *Dasyscypha* sp. isolated from brown leaves in the uppermost layer was also used in some experiments. Besides those isolated from the litter, wood-rotting fungi, *Gyrophana lacrymans* (Fr.) Pat., *Piptoporus betulinus* (Fr.) Karst., *Flammulina velutipes* (Fr.) Sing. and the alien microfungi, *Trichothecium roseum* Link ex Fries from the laboratory stock were used for the comparative study. A number of bacteria were isolated from the litter in varying degrees of decomposition using leaf-litter decoction agar. Among various species of the native bacteria inhabiting in the mouldy leaves, the dominant species is tentatively designated as Bacteria sp. L and the widespread one as Bacteria sp. M. The routine test bacteria, *Escherichia coli* (Mig.) Castell. et Chalm., *Staphylococcus aureus* Rosenbach and *Bacillus subtilis* Cohn emend. Prazm., were employed for the antibacterial screening test.

Fungal cultures in powdered leaves The preparation and sterilization of the powdered beech leaves essentially the same as those described previously. The fungal cultures with the powdered leaves were incubated at 25° to 26°C, unless otherwise stated, in the incubators with approximately 78 per cent relative humidity. In this article, "infected leaves" refer to the powdered leaves artificially moulded in this way.

Chemical analysis A definite amount of the cold-water extract was obtained from the infected leaves by the routine method at appropriate intervals. Prior to the estimation of reducing sugar by the Bertland method, the extracts were purified by a lead acetate method, which is effective in removing tannin and its related substances as well as proteins. Total and water soluble nitrogens were determined by the usual Kjeldahl method. Ammonia was estimated photoelectrically by Nessler's reagent after vacuum distillation. Amino nitrogen was determined as ammonia liberated by the action of ninhydrine following the method of Schlenker (1943).

Chromatographic technique Of the various constituents in the original and the infected leaves, sugars and phenolic compounds were identified by paper chromatography. The papers were developed by the descending method on Tôyô No. 50 filter paper using the one-dimensional technique. Before determining the sugars, the extract purified by the lead acetate method was concentrated *in vacuo* at 40°C and then dissolved in deionized water. The viscous syrup thus obtained was spotted on the papers. The solvent for the sugars was *n*-butanol-acetic acid-water (4 : 1 : 1). The paper was run for four periods each of 24 hours, and after drying, the spots were visualized with benzidine or urea-hydrochloric acid reagent. The phenolic

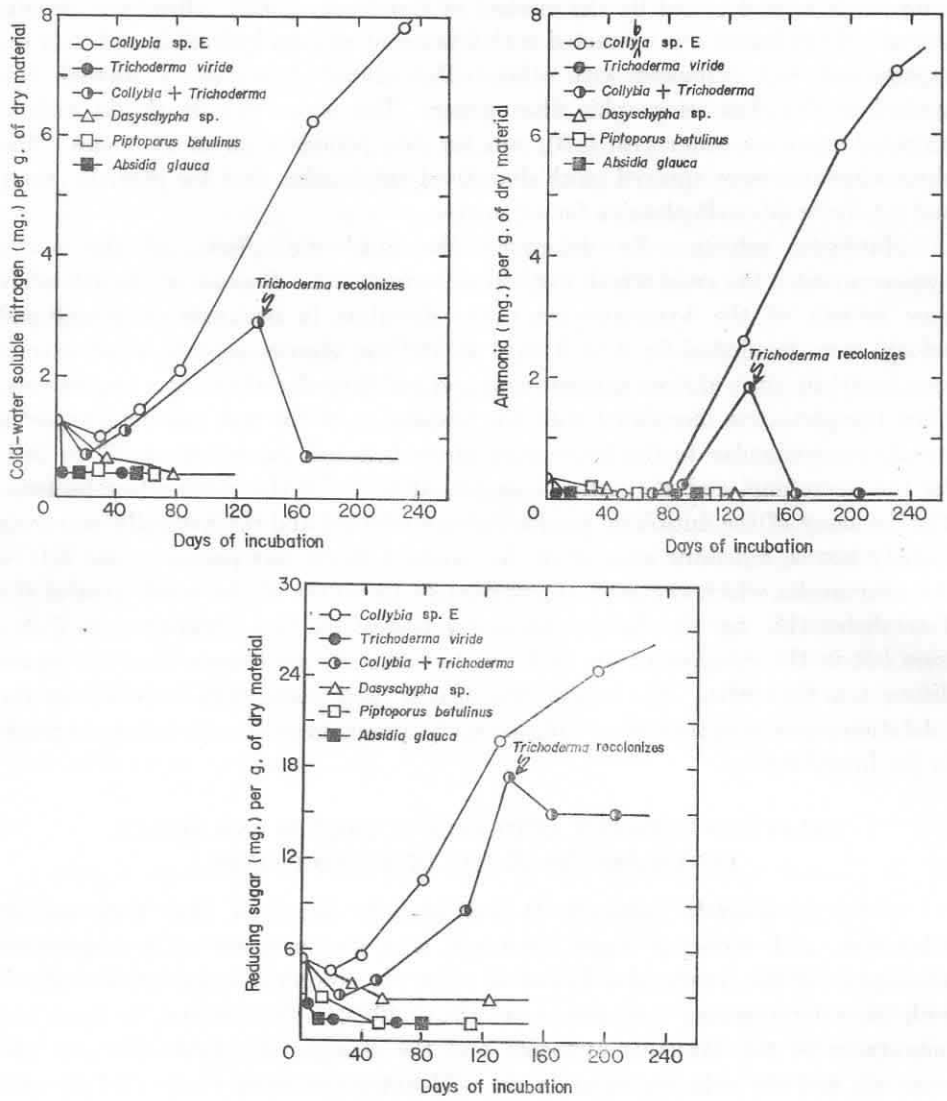
compounds were detected by the method of Henderson (1955). Briefly, a definite amount of the leaves was extracted with 2 per cent sodium hydroxide and then the supernatant was extracted with ether. The extract dissolved in ethanol was applied to the chromatographic filter paper. The paper was developed with *n*-propanol-ammonia-water (16: 1: 3), run for two periods each of 24 hours. The chromatograms were sprayed with diazotized sulphanilic acid for phenolic group and 2,4-dinitrophenylhydrazine for aldehydes.

Antibiotic activity To determine the antibiotic effects of the above hymenomycetes the cross-streak method was employed. Because of the relatively slow growth of the hymenomycetes, the inoculum (a rectangle of precultured colony) were incubated for 3 to 5 days at 26°C on glucose peptone yeast extract agar, nutrient agar, glucose nutrient agar and leaf-litter decoction agar respectively. Then the plate was inoculated with the suspension of the test microorganisms in streaks perpendicular to the hymenomycetous belt and incubated at 25° to 26°C for the microfungi and the native bacteria, at 37°C for the routine test bacteria. For the assay of the antibiotic product of the infected and the naturally occurring mouldy leaves, a definite amount of the moulded leaves was placed on one side of the agar media which occupied approximately two-fifths of the whole area of the Petri dishes (Pl., fig. 2). Before the inoculation of the test organisms, the dishes were left in the refrigerator for 24 hours to make the substances from the leaves diffuse into the media. The results have usually been recorded as the widths of the inhibition zones or in the case of fungi as the test organisms the affected appearances in the fungal colony.

RELATIONS BETWEEN AVAILABLE NUTRIENTS AND FUNGAL DEVELOPMENTS IN THE INFECTED LEAVES

Changes in available nutrients It was already described that water-soluble substances and reducing sugar increases with the progress of decomposition accompanying the intense decolorization when the powdered leaves are inoculated with litter-decomposing hymenomycetes (Saitô 1960). Furthermore, to know the importance of the available nutrients and the changes in soluble nitrogen, the ammonia and the reducing sugar in the cold-water extract of various leaves were estimated. Text-fig. 1 to 3 shows the composition of the leaves in the different stages of decomposition.

As a result of the growth of *Collybia* sp. E, a marked increase occurs in the water-soluble nitrogen. The ammonia and the reducing sugar occur proportionally with the progress of decomposition after a slight decrease at the beginning of decomposition. After more than 100 days of decompositions, a considerable part of the water-soluble nitrogen is in the form of ammonia. In the original brown leaves, only a small fraction of the nitrogen is present in the form of ammonia, that is, 3.41 per cent of the total nitrogen and 13.3 per cent of the cold-water nitrogen; however, at the end of the growth period of *Collybia* sp. E, the ammonia



Text-fig. 1. Changes in cold-water soluble nitrogens in powdered beech leaves infected with fungi singly or in pairs.
Text-fig. 2. Changes in ammonia in powdered beech leaves infected with fungi singly or in pairs.
Text-fig. 3. Changes in reducing sugar in powdered beech leaves infected with fungi singly or in pairs.

nitrogen was found to be about 57.7 per cent of the total nitrogen and about 86.1 per cent of the cold-water nitrogen respectively. These phenomena can be readily explained by the fact that the hymenomycete decomposed not only easily decomposable substances but also resistant materials such as lignins during the

process of decomposition; this leads to the building up of the abundant fungal cells and eventually to the formation of ammonia through autolysis of the hymenomycetous mycelia. The results with *Collybia* sp. F are similar to that of *Collybia* sp. E.

In marked contrast, the wood-rotting hymenomycetes and the cup fungi as well as the microfungi which cause much less decomposition than *Collybia*, behaved quite differently from *Collybia*; there occurs a marked reduction in the water-soluble nitrogen, the ammonia and the reducing sugar at the first stage and in consequence, no accumulation of the available nutrients in the course of decomposition. While, in all the cases of used fungi, the amount of amino nitrogen was proportional to the ammonia but always less as compared with the ammonia.

Collybia may release the reducing sugar from the decomposing leaves, particularly after 60 days or more of the incubation. The reverse is true of the fungi except the litter-decomposing hymenomycetes, since as the results of their growth the reducing sugar in the original brown leaves is almost consumed at the beginning. *T. viride*, *P. betulinus* and *G. lacrymans* are able to utilize cellulose to some extent, but are different from *Collybia* in the fact that there occurs no increase of the reducing sugar.

Thus, based on the differences of changes in the available nitrogen and reducing sugar in the infected leaves, the fungi used here may be divided into two large groups; one of them is litter-decomposing hymenomycetes, the other is a group of microfungi, cup fungi and wood-rotting fungi. By the infection of the latter group, most of the lignin is left intact. Such special ability of hymenomycetes in the decomposition of litter has been intensively studied by Lindeberg (1944, 1947, 1961), Melin (1948) and Norkrans (1950).

Some sugars in the cold-water extract of the original and the infected leaves are identified by paper chromatography. There exist glucose and fructose in beech leaves just fallen in autumn. However, since the original "brown leaves" called in this series of papers remain on the litter layer for about 10 months, with the exception of glucose the sugars seem to be lost owing to leaching and decomposition in the field. The cold-water extract of the leaves infected with *Collybia* showed a strongly stained spot of glucose on the chromatogram, but in the cases of these infected with *T. viride* and *P. betulinus* it was indistinct. Nykvist (1963), who studied leaching and decomposition of various litter extensively, reported that glucose and fructose are found in all litter extracts, xylose mostly and sucrose frequently.

Some factors affecting fungal sequences It is important to clarify the interactions between hymenomycetes and microfungi, with reference to the factors determining the utilization of the available nutrients and the differences in their activities. In this connection, the author already described the successional changes in the mixed culture of hymenomycetes and microfungi with litter; the

initial flare-up of the latter soon declines, then the former becomes predominant, and finally some microfungi grow again depending on the accumulation of the available nutrients due to the hymenomycetous development (Saitô 1960). As will be seen from the results of the dual culture of *Collybia* sp. E and *T. viride* in Text-fig. 1 to 3, the secondary development of *T. viride* (Pl., fig. 3) after about 125 days occurs at the expense of the available nutrients that accumulated at a temperature above 27°C and the evidence for this is the subsequent decrease in the reducing sugar and the ammonia, particularly in the latter. In addition to the case of the coupled inoculation at the beginning of the culture mentioned above, when the leaves were fully infected with *Collybia* prior to the inoculation with the conidiospores of *T. viride*, the colonization of *T. viride* is successful over 27°C. If the mixed culture is kept below 25°C, however, the secondary appearance of *T. viride* does not occur, notwithstanding the accumulation of the available nutrients. At 26° to 27°C, the reappearance of *T. viride* was delayed considerably. Therefore, it must be kept in mind that the secondary development of microfungi occurs under limited conditions, such as within a certain range of temperature. This seems to be due to the fact that the optimum temperature for the growth of *Collybia* is lower than that of *T. viride*.

The recolonization of microfungi takes place not only in the combinations of *Collybia* sp. E and *T. viride*, but also in those of *Collybia* sp. F and *T. viride*, and of *Collybia* sp. F and *Penicillium lapidosum*, as stated in the previous work (1960). The initial periods of the recolonization and their activities appear to be related to the differences in competitive saprophytic ability of each fungus. For example, *P. lapidosum* is inferior to *T. viride* in the competitive saprophytic ability, so that it shows only a sparing growth after a pronounced depression in the activity of the hymenomycete.

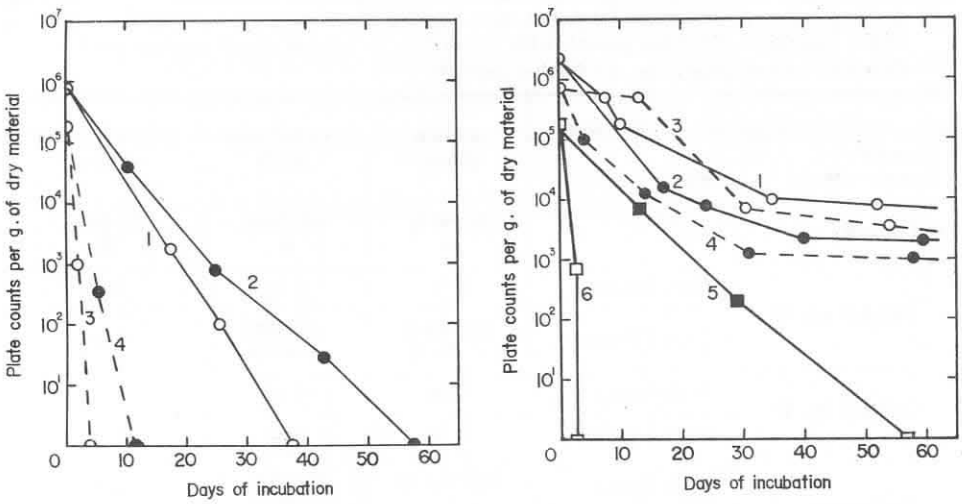
According to Griffiths and Siddiqi (1961), the temperature markedly affects antagonism towards *Fusarium culmorum* by *T. viride* in sterilized soil; at temperatures below 10°C *F. culmorum* is wholly dominant, whereas at higher temperatures the situation is reversed. Taylor (1964) considered that the temperature had important effects on active saprophytic colonization by the two root colonizers, *Fusarium oxysporum* and *Cylindrocarpon radicicola*. In the present work it is also concluded that temperature is an important factor influencing competitive saprophytic colonization, apart from the changes in the nutritional status.

THE FATE OF MICROORGANISMS INOCULATED IN THE HYMENOMYCETE-INFECTED LEAVES

Little is known of the interactions between hymenomycetes and soil microorganisms in natural habitats, except for the notes by Warcup (1951a) and Chalvignac, Tysset and Pochon (1959). Warcup noticed the very interesting fact that the mycelial zones of certain basidiomycetes contain a restricted population of microfungi, fewer both in the numbers of species and of colonies than those in

the normal soil. This tendency was also seen in the mouldy leaves in the beech litter layer (Saitô 1956). Moreover, in view of the fact that *A. glauca* dies out when inoculated in the powdered leaves together with hymenomycete (Saitô 1960), it was suggested that these hymenomycetes may have a lethal action on some fungi. The following experiments were made to confirm such an antimicrobial action of the infected leaves.

Inoculation of the spores and the pre-developed hyphae of microfungi First, a definite amount of homogeneous spore suspensions and the predeveloped hyphae (Stevenson 1956) were added to the leaves incubated for 25 to 30 days after being infected with *Collybia* and also to those incubated for 7 to 10 days after being infected with each of *G. lacrymans*, *P. betulinus* and *F. velutipes*. And then the survival of their inocula was counted by the dilution plate method at appropriate intervals. For preparing the pre-developed hyphae, the spore suspension of *A. glauca* was added to glucose peptone mineral culture solution and incubated at 28°C for 8 hours and that of *T. viride* for 9 hours by shaking culture to produce vegetative growth of c. 50μ to 150μ long. These results are shown in Text-fig. 4 and 5.



Text-fig. 4. Survival of fungal spores and pre-developed hyphae added to powdered beech leaves infected with hymenomycete: (1) *A. glauca* spores inoculated in *Collybia* sp. E-infected leaves, (2) *A. glauca* spores inoculated in *Collybia* sp. F-infected leaves, (3) *A. glauca* pre-developed hyphae inoculated in *Collybia* sp. E-infected leaves, (4) *A. glauca* predeveloped hyphae inoculated in *Collybia* sp. F-infected leaves.

Text-fig. 5. Survival of fungal spores and pre-developed hyphae added to powdered beech leaves infected with hymenomycete: (1) *T. viride* spores inoculated in *Collybia* sp. E-infected leaves, (2) *T. viride* spores inoculated in *Collybia* sp. F-infected leaves, (3) *T. viride* pre-developed hyphae inoculated in *Collybia* sp. E-infected leaves, (4) *T. viride* pre-developed hyphae inoculated in *Collybia* sp. F-infected leaves, (5) *A. glauca* spores inoculated in *Piptoporus betulinus*-infected leaves, (6) *T. roseum* spores inoculated in *Collybia* sp. E-infected leaves.

When the spores and the pre-developed hyphae are added to the leaves infected with each of *Collybia* and *P. betulinus*, there is seen a gradual disappearance or a definite diminition in the numbers. *A. glauca* is highly susceptible to *Collybia* sp. E, particularly in the form of the pre-developed hyphae. As a rule, *T. viride*, both spore and pre-developed hyphae, is most resistant and detected to have the vitality even at the end of about 60 days incubation. The spore of *T. roseum* is more sensitive than that of *A. glauca*. The leaves infected with *G. lacrymans* or *F. velutipes*, permit the germination of the inoculated spores and the following conspicuous growth of mycelia.

For studying the lethal effect of the hymenomycetes in more detail, the direct microscopic technique was also employed. The germination rates of the spores and the characteristic morphological changes of the germ tube were pursued at some intervals with the infected leaves. These observations were

Table 1

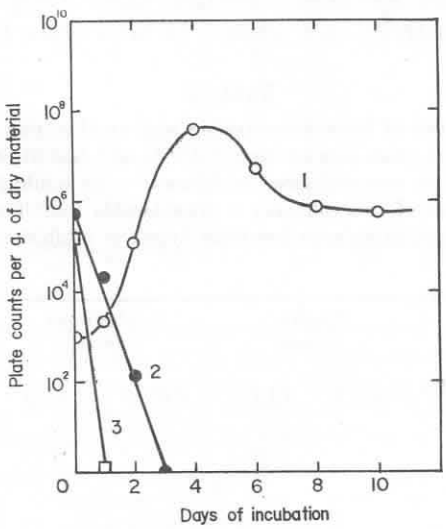
Germination rates of fungal spores inoculated and the resulting hyphal development in the powdered leaves infected with hymenomycetes, both fresh and heated leaves. Hyphal growth was estimated as follows: ### healthy and very extensive hyphal development; ## almost healthy but slow hyphal development; # abnormal and slight inhibition of hyphal growth with distortion; + abnormal and hyphae swollen distorted or protuberances, no further growth.

inoculated hymenomycete \ test fungi / leaves		*Absidia glauca*	*Trichoderma viride*	*Trichothecium roseum*
Control		30-60 % ###	40-70 % ###	50-70 % ###
Collybia sp. E	fresh leaves	0 %	0 %	0 %
	heated leaves	0.5-10 % + to #	5-20 % + to #	0 %
Collybia sp. F	fresh leaves	0 %	0 %	0 %
	heated leaves	0 %	0 %	0 %
Piptoporus betulinus	fresh leaves	0.01-0.1 % +	25-40 % + to #	0 %
	heated leaves	0.05-0.2 % +	30-50 % #	0 %
Gyrophana lacrymans	fresh leaves	1-3 % #	80-90 % ###	40-60 % ##
	heated leaves	1-10 % # to ##	90-95 % ##	60-80 % ##
Flammulina velutipes	fresh leaves	1-6 % #	60-70 % ##	50-60 % ##
	heated leaves	5-15 % #	70-90 % ##	60-70 % ##

performed on the leaves treated with hot water of 45° to 50°C for 24 hours as well as on the freshly infected leaves. This heat treatment, a kind of pasteurization, results in a complete sterilization of hymenomycetous mycelia. These data are given in Table 1.

A failure or a low percentage of germination is recorded with both fresh and heated leaves infected with each of *Collybia* and *P. betulinus*, and thenafter the inhibitory effects such as stunting, distortion, excessive branching and formation of hyphae protuberances were noticed in the course of the abnormal developments of the germ tubes and hyphae. The same tendency was also seen in those inoculated with pre-developed hyphae.

On the contrary the relatively high percentages of germinations in the spores of *T. viride* and *T. roseum* were obtained in the leaves infected with *G. lacrymans* and *F. velutipes*. The majority of the spores grows into their healthy hyphae though slowly, and eventually results in sporulation. The effect of the heat treatment varies from species to species. The decline of the inhibitory action is found to some extent in the cases of *Collybia* sp. E and *P. betulinus*, and in consequence more or less germination occurs in *A. glauca* and *T. viride*. The reverse is true of *Collybia* sp. F, much inhibition being distinctly observed in the heated and infected leaves. Thus, it is very likely that the presence of active mycelia is not essential to the inhibitory action but is to the chemical deterioration. And these findings suggest that the killing of microfungi is not due to the shortage of the nutrients and the accumulation of carbon dioxide, but to the toxic substances produced by the fungus



Text-fig. 6. The fate of bacteria added to powdered beech leaves infected with *Collybia* sp. E: (1) Bacteria sp. L inoculated in *Collybia* sp. E-infected leaves, (2) Bacteria sp. M inoculated in *Collybia* sp. E-infected leaves, (3) *E. coli* inoculated in *Collybia* sp. E-infected leaves.

or the decaying products in the leaves.

Inoculation of bacteria A definite amount of bacterial suspension were added to the leaves of pH 3.8 after 25 to 30 days of incubation with *Collybia* sp. E and then the bacterial number was counted at a certain interval by the plate count method.

E. coli and a vast majority of isolates from the litter, such as Bacteria sp. M, disappear rapidly as shown in Text-fig. 6. On the other hand Bacteria sp. L is able to reproduce to some extent in the infected leaves. Accordingly, the predominated bacteria is characterized by the ability of growing in the infected leaves which have the acidity of as low as pH 3.8 and the lethal effect. Whether the marked decrease in pH or the antibiosis was responsible for the disappearance of the inoculated bacteria can not be decided at present. Further work in this connection is in progress.

ANTIBIOTIC ACTIVITY OF HYMENOMYCETES IN THE CULTURE MEDIA AND THE POWDERED LEAVES

A number of works on the antibiotic production of basidiomycetes were reported by Wilkins (1948) and many others, but little is known of the antibiotic production in the forest litter. It was already described that the hymenomycetes isolated form the beech litter have a bacteriostatic action (Saitô 1958). This section deals with further study on the antibiotic activity of the infected leaves.

Antibiotic activity against microfungi As shown in Table 2, *Collybia* sp. E exhibits a slight inhibiting action against all of the test fungi on glucose peptone yeast extract agar and leaf-litter decoction agar. *Collybia* sp. F is inferior to *Collybia* sp. E in the activity as a whole. In both cases, the larger antagonistic

Table 2

Antifungal activities of litter-decomposing and wood-rotting hymenomycetes on glucose peptone yeast extract agar (GPYA) and leaf-litter decoction agar (LLDA). Inhibition was estimated as follows: – no inhibition; + slight inhibition on contact of two coliones; ++ considerable inhibition on contact of two colonies; +++ inhibition zone less than 5 mm; +++ inhibition zone more than 5 mm.

test fungi / media / hymenomycete	*Absidia glauca*		*Trichoderma viride*		*Trichothecium roseum*	
	GPYA	LLDA	GPYA	LLDA	GPYA	LLDA
Collybia sp. E	+	+++	+	+	+	+++
Collybia sp. F	–	++	–	–	++	+++
Piptoporus betulinus	++		+		+++	
Gyrophana lacrymans	–	–	–	–	–	–
Flammulina velutipes	–	–	–	–	–	–

spectra are seen in leaf-litter decoction agar than glucose peptone yeast extract agar. *P. betulinus* which is known to produce the antibacterial substance called ungulinic acid, was found also to show an antifungal activity. *G. lacrymans* and *F. velutipes* did not show the inhibitory activity against all the fungi. The hymenomycetes that proved to have the lethal activity in the infected leaves as seen in Text-fig. 4 and 5 exerted a fairly inhibitory action on the agar media.

The antifungal activities of the leaves infected with *Collybia*, both fresh and heated, are presented in Table 3. As a rule, the inhibitions of the infected

Table 3

Antifungal activities of the infected leaves with each of litter-decomposing and wood-rotting hymenomycetes, fresh and heated leaves, using glucose peptone yeast extract agar as assay media. Inhibition was estimated as follows: − no inhibition; + very slight inhibition on contact of two colonies; ++ slight inhibition on contact of two colonies; +++ considerable inhibition on contact of two colonies; ||| inhibition zones less than 5 mm.

inoculated hymenomycete \ test fungi / leaves		*Absidia glauca*	*Trichoderma viride*	*Trichothecium roseum*
Collybia sp. E	fresh leaves	+	+	++
	heated leaves	−	−	+
Collybia sp. F	fresh leaves	−	−	+++
	heated leaves	++	++	|||
Piptoporus betulinus	fresh leaves	+	−	++
	heated leaves	−	−	+
Gyrophana lacrymans	fresh leaves	−	−	−
	heated leaves	−	−	−
Flammulina velutipes	fresh leaves	−	−	−
	heated leaves	−	−	−

leaves are less against *A. glauca* and *T. viride* than that in glucose peptone yeast agar. *T. roseum*, however, was somewhat more susceptible to the action of the infected leaves. The heat treatment of the infected leaves results in the reduction of inhibition in the cases of *Collybia* sp. E and *P. betulinus* but increases the inhibition in the case of *Collybia* sp. F.

Antibiotic activity against bacteria Antibacterial spectra on three kinds of agar media are presented in Table 4. The results almost agree with the ones of the previous cases, but the degree of inhibition varies a little according to the difference of the media. Generally, both species of *Collybia* showed the strongest inhibition in glucose peptone yeast extract agar, less in glucose nutrient agar, but no appreciable inhibitory effect in nutrient agar. The reason may be that there

Table 4

Antibacterial spectra of *Collybia* on glucose yeast extract agar (GPYA), nutrient agar (NA) and glucose nutrient agar (GNA), as indicated with the widths (mm) of inhibition zones.

test bacteria / antibiotic producer / media	*E. coli*			*B. subtilis*			*S. aureus*			Bacteria sp. L		
	GPYA	NA	GNA	GPYA	NA	GNA	GPYA	NA	GNA	GPYA	NA	GNA
Collybia sp. E	15-19	0	0	20-22	0	13-15	20-30	0	13-18	0	0	0
Collybia sp. F	7-12	0	0	8-13	0	2-3	15-23	0-3	5-6	0	0	0

occurs not only the poor growth of both species of *Collybia* but also the depression of their inhibitory activities in the nutrient agar without sugar. The native bacteria, Bacteria sp. L and Bacteria sp. M are not sensitive to these hymenomycetes. Of 42 bacterial isolates from the litter layer, only two species are susceptible to *Collybia*. But these senistive bacteria are not the main members in the natural population.

Similarly, the leaves infected with *Collybia* and incubated for 30 to 45 days, both fresh and heated, showed the inhibition zones (Pl., fig. 2) against test bacteria in glucose peptone yeast extract agar and glucose nutrient agar, as seen in Table 5. The antibiotic activity varies considerably from media to media as already described. The difference of behaviour between the antibiotics produced in the leaves and in the agar media, is quantitative rather than qualitative except for the effect against *E. coli*. On glucose nutrient agar *E. coli* was not susceptible to *Collybia* but the leaves infected with *Collybia*.

In this case also the heat treatment of the *Collybia* sp. E-infected leaves brought about the reduction of the antibiotic activity, while that of *Collybia* sp. F the rise

Table 5

Antibacterial spectra of the leaves infected with *Collybia*, as indicated with the widths (mm) of inhibition zones, using glucose peptone yeast extract agar (GPYA) and glucose nutrient agar (GNA) as assay media.

test bacteria / antibiotic producer / infected leaves / media	*E. coli*		*B. subtilis*		*S. aureus*		Bacteria sp. L	
	GPYA	GNA	GPYA	GNA	GPYA	GNA	GPYA	GNA
Collybia sp. E — fresh leaves	5-6	4-5	10-12	8-10	12-15	12-13	0	0
Collybia sp. E — heated leaves	4-5	3-4	6-7	4-5	12-14	6-7	6-7	5-7
Collybia sp. F — fresh leaves	7-8	3-4	8-10	7-8	4-8	3-4	0	0
Collybia sp. F — heated leaves	14-15	8-9	16-19	16-17	11-13	11-12	9-10	8-10

of the activity. It is to be noted that the native bacteria represented by Bacteria sp. L and Bacteria sp. M are inhibited by the infected leaves only after the heat treatment. Though this scarcely happens in the natural environments, the effect of the heating is of interest.

ANTIBIOTIC ASSAY OF THE MOULDY LEAVES IN THE NATURAL HABITATS

It is necessary to know whether the antibiotics produced by the hymenomycetes are effective *in situ* against their neighbouring microbes. The mouldy leaves in which each of *Collybia* densely overgrows were taken from the litter layer under beech forests. These were placed directly on the three kinds of agar media mentioned above and examined by the cross-streak method. To prevent the growth of vigorous associated bacteria from the leaves, several modified assay methods were also applied.

In no case was any antibiotic activity detected in the naturally occurring moudly leaves. The explanation for the absence of the inhibitory action may be that the antibiotics produced are readily destroyed by the concomitant vigorous microbial population and then by the inhibition of the hymenomycetous growth and of the antibiotic synthesis which occurs by the antagonistic actions of the competitors. In fact, a vigorous growth of bacteria associated with a large amount of hymenomycetous mycelia was noticed at all stages in relation to the decomposition of the mouldy leaves, as mentioned in the previous paper (Saitô 1958).

There is an evidence of the antibiotic production of fungi grown in straw and viable seeds by Wright (1956) who found sensitive bioassay using the chromatographic method. The effectiveness of the antibiotics produce *in situ*, however, still remains obscure for the surrounding microbial flora. Further work along this line would throw light upon the nature of the interaction among the microorganisms in plant debris.

From these results, it is probably unreasonable to consider that the antibotic activity of the hymenomycetes may lead to the decrease of the population of the microfungi in the mouldy leaves in the natural hatitats. The reduction in fungal number may not be due to the antibiosis and the competition for food, but to some other competitive factors or after-effect by explosive development of the acid tolerant bacteria. As soon as the active colonization of *Collybia* ceases in the mouldy leaves and the yellowish leaves, there occurs a rise in numbers of the microfungi.

RELEASE OF PHENOLIC COMPOUNDS FROM BEECH LEAVES DURING DECOMPOSITION BY *COLLYBIA*

By means of paper chromatography Henderson (1955) reported that aromatic compounds such as vanillic acid and syringic acid were released from the spruce and birch sawdust by white-rot fungi.

When *Collybia* was grown over a period of about three months in the powdered

leaves pretreated with alcohol-benzene, it clearly released vanillic and syringic acid which could be isolated by alkaline extraction of the infected leaves. At the same time, vanillin and syringaldehyde were also detected in the same extract, while only faintly in the control. It is evident that these aromatic compounds identified here are the degradation products of lignin by *Collybia*. On the other hand, in those infected with *A. glauca*, *T. viride* and *P. betulinus* the spots of faint reaction were detected with regard to both acids and aldehydes.

So far as the degradation products of lignin are concerned, there seems to be no differences between the wood by white rot fungi and the leaves by litter-decomposing hymenomycetes.

According to Henderson and Farmer (1955), a large number of soil fungi are able to decompose the phenolic compounds relating to lignin. And Henderson (1955, 1960) suggested that the phenolic compounds released by lignin-decomposer can be rendered available to the microfungi in the successional stages under natural conditions. Apart from the formation of the phenolic compounds in the decomposition of lignin, Melin (1944), Gerd (1956) and Harley (1959) stated that the phenolic compounds and their related substances in litter are of considerable importance for the gowth of a microorganism. Burges (1965) assumed that the phenolic materials in pine litter act as efficient inhibitors to many species of bacteria, in the course of microbial succession. Lingappa and Lockwood (1962) postulated that lignin monomers are fungitoxic in relation to soil fungistasis. Further studies are needed to find how much the phenolic substances can be detected from the moudly leaves.

DISCUSSION

Recent approaches to the fungal ecology are made through a study of the ecological grouping of the fungi defined by Garrett (1951, 1956) as well as the exploitation of a new fungal world by Warcup (1957, 1962) and others. The types of microbial coaction were discussed by Park (1960), Jackson (1965) and others. In the present study, the microfungi and the litter-decomposing hymenomycetes belong to different substrate groups; in other words, they are free from the competition for the similar requirements, because they do not occur in the same niche theoretically. According to Clark (1965), among the active microbial species in soil, food specialization makes possible the existence of a large number of ecological niches within any given habitat. This may well be true. Nevertheless, some examples of the coactions were clearly demonstrated between hymenomycetes and microfungi. The relation, however, is not likely in active competition where an ecological niche overlap partially.

In the dual culture of hymenomycetes and microfungi with the beech leaves, *in vitro*, *Collybia* create not only excellent nutritional conditions, but also the microenvironment unsuitable for the growth of other species by the production of antibiotics or by changes in the acidity of the decomposing leaves. The en-

vironmental factors, both abiotic and biotic, may be responsible for the actual exhibition of these two conflicting microbial reactions in the natural habitat. As far as the mouldy leaves are concerned the increase of the available nutrients and the fall in the acidity account for the findings in the field. As a result there are found the tendency of a gradual rise in the fungal number and a marked increase in the acid tolerant bacteria. These developments of the subsequent microbial population may be regarded as a recolonization in a wide sense.

In this connection, it is of interest that, according to field observation, the massive patches of *T. viride* (Pl., fig. 4), from 3 to 5 cm in diameter, are found on the old mouldy leaves though extremely rare. This colonization, however, may be almost confined to the highly moist conditions. The flare-up of *T. viride* may possibly be thought to result from the loss of viability of the hymenomycetes due to the excessive moisture and the cessation of their active growth. Warcup (1951b) and Evans (1955) reported that the dominant recolonizing fungus is *T. viride* when the soil is sterilized by formalin. They and Saksena (1960) found that *T. viride* also recolonizes soil fumigated by a low dosage of carbon disulphide, but more resistant fungi replaced it at higher dosage. There is an opinion by Garrett (1957, 1958, 1965) that the recolonization of fumigated soil by *T. viride* is likely to occur only under a soil condition such as a relatively high soil temperature, good aeration and acidic condition. Thus, the recolonization of *T. viride* may be due to its high growth rate and competitive ability and also to its potentiality of parasitism against other fungi.

T. viride employed in this series was the only one of many isolates obtained from the litter layer. This isolate showed a slight inhibition against *B. subtilis* and *S. aureus* by the cross-streak method, but the culture filtrates failed to demonstrate the antibiotic activity. As Webster (1964) and Webster and Lomas (1964) pointed out, there is a confusion between *Trichoderma* and *Gliocladium* in respect to the identification and the antibiotic production. In addition, this strain of *Trichoderma* was not morphologically identical with the *Gliocladium*-type fungi.

SUMMARY

1. The increase in water-soluble nitrogen and reducing sugar, consisting mainly of ammonia nitrogen and glucose respectively, is recorded with the progress of decomposition when the powdered beech leaves are inoculated with litter-decomposing hymenomycetes *Collybia*. In contrast to them, microfungi, cup fungi and wood-rotting hymenomycetes lead to a decrease in the available nutrients in decomposing leaves.

2. In mixed cultures of microfungi and litter-decomposing hymenomycetes, the recolonization of some primary colonizer develops at the expense of the loss of viability of the secondary colonizer as well as by the accumulation of these available nutrients. The temperature is also responsible for the success of the recolonization because there is a difference in optimum temperature between them.

3. *Collybia* exert a fairly severe inhibition against the routine test bacteria and a slight inhibition against microfungi on the culture media. Likewise, the leaves deliberately infected with *Collybia*, show more or less inhibitory activity against bacteria and microfungi. In some cases, the effect of the antibiotics produced in the infected leaves is somewhat different from that in the agar media.

4. The killing of the spores and the hyphae of microfungi added to the leaves infected with *Collybia* are due to their antibotic production.

5. No appreciable antibiotic activity was detected in the naturally occurring mouldy leaves accompanied with vigorous bacterial population.

6. In the process of the degradation of lignin in the *Collybia*-infected leaves, vanillic acid and syringic acid are released considerably, then vanillin and syringaldehyde are detected.

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EXPLANATION OF PLATE

Fig. 1. The fruit-bodies of *Collybia* sp. E on the beech litter.

Fig. 2. The antibacterial spectra of the *Collybia* sp. E-infected powdered leaves: (A) *Collybia* sp. E-infected powdered leaves, (a) *B. subtilis*, (b) *S. aureus*, (c) *E. coili*, (d) Bacteria sp. L.

Fig. 3. The recolonization of *Trichoderma viride* on the *Collybia* sp. E-infected powdered leaves in flask: (a) secondary development of *T. viride* when combined with *Collybia* sp. E, (b) powdered beech leaves infected with *Collybia* sp. E singly.

Fig. 4. The recolonization of *Trichoderma viride* on the old mouldy leaves infected with *Collybia* sp. E in the natural habitat.


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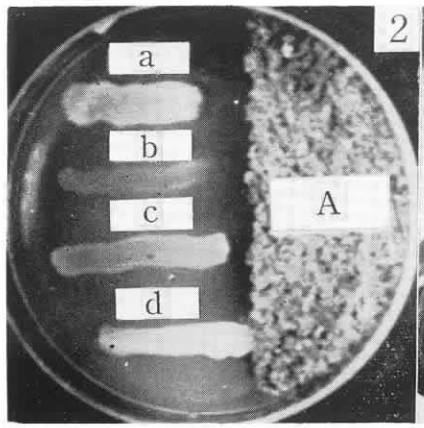
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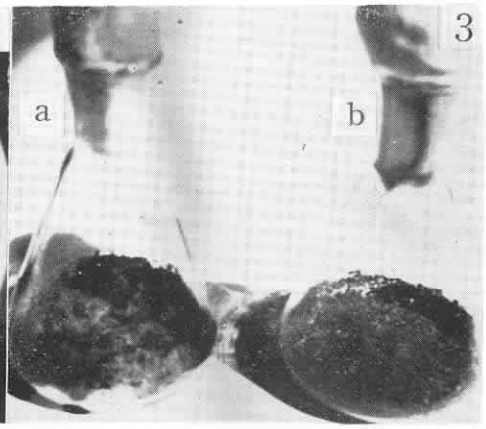
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